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Lactate regulates myogenesis in C2C12 myoblasts in vitro

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Abstract Satellite cells (SCs) are the resident stem cells of skeletal muscle tissue which play a major role in muscle adaptation, e.g. as a response to physical training. The aim of this study was to examine the effects of an intermittent lactate (La) treatment on the proliferation and differentiation of C2C12 myoblasts, simulating a microcycle of high intensity endurance training. Furthermore, the involvement of reactive oxygen species (ROS) in this context was examined. C2C12 myoblasts were therefore repeatedly incubated for 2 h each day with 10 mM or 20 mM La differentiation medium (DM) and in some cases 20 mM La DM plus different antioxidative substances for up to 5 days. La free (0 mM) DM served as a control. Immunocytochemical staining, Western blot analysis and colorimetric assays were used to assess oxidative stress, proliferation, and differentiation. Results show that La induces oxidative stress, enhances cell-cycle withdrawal, and initiates early differentiation but delays late differentiation in a timely and dose-dependent manner. These effects can be reversed by the addition of antioxidants to the La DM. We therefore conclude that La has a regulatory role in C2C12 myogenesis via a ROS-sensitive mechanism which elicits implications for reassessing some aspects of training and the use of nutritional supplements.

Abbreviations: La, lactate; ROS, reactive oxygen species; DM, differentiation medium; AA, ascorbic acid (vitamin C); NAc, N-Acetyl-L-cysteine; LA, linolenic acid; SC, satellite cell; Pax7, paired box transcription factor 7; Myf5, myogenic factor 5; MyoD, myogenic determination protein; MHC, myosin heavy chain; H_2O_2, hydrogen peroxide; MAPK, mitogen-activated protein kinase; PM, proliferation medium; BSA, bovine serum albumin; ICC, immunocytochemical staining; 8-epi-PGF2α, 8-epi-prostaglandin F_2 alpha; act. Casp-3, activated Caspase-3; DAPI, 4′,6-Diamidino-2-phenylindole; DAB, 3′,3′-Diaminobenzidine; WB, Western blot; BrdU, Bromodeoxyuridine; SOD, superoxide dismutase; GPx, glutathione peroxidase; Cat, Catalase; a.u., arbitrary units (grey values).

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Introduction

Skeletal muscle tissue contains so called satellite cells (SCs) which lie quiescent underneath the basal membrane and make up the necessary stem cell pool for myogenesis. In response to muscle injury SCs can be activated to become proliferating myoblasts that differentiate in order to repair muscle tissue. During this process, SCs and myoblasts underlie a strict sequential expression pattern of different transcription factors and structural muscle proteins (Yun and Wold, 1996). These can be used as markers to study the proliferation and differentiation behaviour of this cell population in vitro and are extensively reviewed in Yablonka-Reuveni et al. (2008). Briefly, a transient reduction of paired box transcription factor 7 (Pax7) expression coincides with the withdrawal from the cell cycle and transition of the proliferation into the differentiation phase. Whereas the myogenic determination protein (MyoD) and myogenic factor 5 (Myf5) are soon expressed following activation of SCs, myogenin is expressed at a later stage marking the commitment to differentiation. The end-terminal differentiation coincides with myosin heavy chain (MHC) expression, marking sarcomeric assembly.

The involvement of SCs in muscle regeneration has been well characterised. However, their role in mediating exercise-induced adaptations remains less clear. Most emphasis has been placed upon elucidating the contribution of SCs in skeletal muscle adaptation in response to strength training, where muscle fibre hypertrophy is the main adaptation. Only few studies have looked at the role of SCs as a response to a training regimen that does not generally induce hypertrophy, i.e. endurance or aerobic interval training. Results from an animal study (Umnova and Seene, 1991) and two human studies (Charifi et al., 2003; Verney et al., 2008) have implied that endurance type training enhances the satellite cell pool. Furthermore, the literature suggests that this enhancement is influenced by the intensity rather than by the duration of exercise (Kurosaka et al., 2012). This would also explain the results of Snijders et al. who could not find a change in the satellite cell content in diabetes type 2 patients following a 6-month continuous, endurance-type exercise programme (Snijders et al., 2011). Their workload corresponded to about 75% VO_{2max}, whereas the other studies used intermittent protocols with intensities around 75–95% VO_{2peak} (Charifi et al., 2003) or 75–95% HR_{max} (Verney et al., 2008). Moreover, one study provides evidence that SCs are not only activated but also have a role in functional adaptations following non-hypertrophic training such as skeletal muscle fibre remodelling (Joanisse et al., 2013).

Taken together, the current literature suggests that in response to aerobic training SCs are activated in an intensity-dependent manner and play an active role in non-hypertrophic skeletal muscle adaptation.

The challenge arising with this hypothesis is to identify the stimuli that occur at different concentrations depending on the exercise intensity. One possible candidate is Lactate (La). It has long been considered a metabolic waste product and the cause of decrease in muscle pH and hence muscle fatigue. This idea has changed massively in the past. Now it is known that La is rather an intermediate of glucose metabolism, acting as an energy substrate and a gluconeogenic precursor. It has also been termed Lactormon for its signalling properties, inducing gene expression necessary for skeletal muscle adaptation (Brooks et al., 2008) as Hashimoto et al. could demonstrate that La increases MCT1 and PGC1α mRNA content (Hashimoto et al., 2007). Further investigations by the same group led to the conclusion that most likely these effects are mediated by reactive oxygen species (ROS) as hydrogen peroxide (H_2O_2) has been shown to increase and that the adaptations caused are most likely generated via a vast oxygen-radical sensitive network and subsequent mitogen activated protein kinase (MAPK) signalling (Hashimoto et al., 2007).

The aim of the study was to investigate the influence of a simulated microcycle of intensive endurance training on the proliferation and differentiation of myoblasts by intermittently treating C2C12 cells with physiologically relevant La concentrations. Furthermore, we wanted to investigate if La increases oxidative stress within the cells, and whether this increase is – at least partly – responsible for the observed La-effects.

Materials and methods

C2C12 Cell culture and treatment

C2C12 mouse myoblasts (Yaffe and Saxel, 1977a), obtained from the DSZM Braunschweig, Germany, were kept in cell culture flasks (BD Falcon, Bedford, USA) at 37 °C and 5% CO_2 in proliferation medium (PM) consisting of DMEM, 1% penicillin-streptomycin, 4 mM glutamine, 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate (all from Invitrogen, Karlsruhe, Germany), and 20% foetal calf serum (PAA, Pasching, Austria). For experimental procedures, cells were plated on gelatine-coated (0.1% in PBS) glass cover slips or Petri dishes at a density of 10,000 cells per cm^2. After plating, cells were kept in proliferation medium until 80–90% confluence was reached. Thereafter, medium was switched to differentiation medium (DM) containing DMEM, 1% penicillin/streptomycin, 4 mM glutamine, 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate, and 4% horse serum (all from Invitrogen, Karlsruhe, Germany). L-sodium lactate (Sigma-Aldrich, Steinheim, Germany) was added to give different concentrations of 10 mM and 20 mM La DM. These levels were observed in human blood and muscle after vigorous exercise and for these reasons have been used previously in the literature (Hashimoto et al., 2007; Ohkuwa et al., 1984; Cheetham et al., 1986; Jung et al., 2004). Cells were incubated intermittently with La DM for up to 5 days to create a more realistic training situation where La levels are only elevated for a certain amount of time compared to continuous treatment. During the experiment medium was changed to La DM (10, 20 mM) and control DM (La: 0 mM) for 2 h at the same time each day. Afterwards all cells were given control DM. After the initial experiments on the dose-dependency of the La effect, experiments were repeated adding different antioxidative agents to the 20 mM La DM. These were L-ascorbic acid (AA; 100 μM), N-Acetyl-L-cysteine (NAc; 5 mM), and linolenic acid (LA; 5 μM; all from Sigma-Aldrich, Steinheim, Germany). All experiments were repeated at least twice in independent runs.

Immunocytochemistry (ICC)

Cells were fixed before start of treatment (pre) and on days 1, 2, 3, and 5 by 15 min incubation with 4% paraformaldehyde (Roth Chemicals, Karlsruhe, Germany) in DPBS

(Invitrogen, Karlsruhe, Germany) followed by 3 washing steps with DPBS. Thereafter, cells were stored at 4 °C until undergoing immunocytochemical staining. For the detection of ROS (see below) cells were treated only once and PFA-fixed immediately after the treatment. Cells treated to demonstrate the effects of La and antioxidants were fixed before (pre) and after 5 days of treatment. After fixation and storage at 4 °C cells were permeabilized with 0.5 M ammonium chloride solution in PBS containing 0.25% Triton X-100 for 10 min followed by 4 washing steps with PBS. For cells undergoing DAB staining an incubation phase with 0.6% H_2O_2 in 80% methanol to eliminate endogenous peroxidase activity followed. In order to avoid unspecific binding, cells were blocked with 5% bovine serum albumin (BSA) in PBS for one hour. Primary antibody was diluted in 0.8% BSA and cells were incubated at 4 °C overnight. Antibodies used were anti-Ki67 (1:250; Abcam, Cambridge, UK), anti-active-Caspase-3 (act. Casp-3; 1:500; BD Pharmingen, Franklin Lakes, NJ, USA), anti-8-epi-prostaglandin F2α (8-epi-PGF2α, also known as 8-isoprostane or 15-f_{2t}-isoprostane; 1:1500; Oxford Biomedical Research, Oxford, MI, USA), anti-Pax7 (1:250; Neuromics, Edina, NM, USA), anti-Myf5 (1:250; GeneTex, Irvine, CA, USA), anti-F5D (1:500), and anti-Mf20 (1:500; both from Developmental Studies Hybridoma Bank, Iowa City, IA, USA). The following day, cells were washed 4 times with PBS and treated with secondary antibody solution. Ki67, act. Casp-3, and 8-epi-PGF2α incubated cells were treated with goat-anti-rabbit streptavidin-conjugated secondary anti-body (1:400 in PBS), followed by 4 washing cycles and incubation with horseradish peroxidase (1:150 in PBS; both DAKO, Glostrup, Denmark). Diaminobenzidine (DAB) was used as a detection system for the signal. Those cells treated with the other antibodies were incubated with Alexa488 coupled secondary antibody (1:1000 in PBS; Invitrogen, Eugene, OR, USA) for 60 min, followed by a washing interval and stained with DAPI (Sigma-Aldrich, Steinheim, Germany) for 5 min and following another washing interval mounted on microscope slides using Aquapolymount (Polysciences Inc., Warrington, PA, USA). DAB stained cells were dehydrated using ethanol and xylol, and mounted with Entellan® (Merck KgaA, Darmstadt, Germany).

Analysis for Ki67, Pax7, Myf5, F5D and Mf20

Cover slips were analysed using a Zeiss Axiovert 200 M (Carl Zeiss AG, Jena, Germany). For every condition 20 pictures of cells with a 20× objective were taken and total nuclei number counted using Image J software (National Institute of Health, Bethesda, MD, USA). Ki-67, Pax7, Myf5, F5D or Mf20 positive nuclei were counted manually. The ratio number of Ki67/Pax7/Myf5/F5D/Mf20 positive cells to total number of nuclei was calculated.

Analysis for activated Caspase-3 and 8-epi-PGF2α

Act. Casp-3 and 8-epi-PGF2α staining were analysed using densitometry. Pictures at 20× magnifications were taken and at least 200 cells per condition were analysed for staining intensity using Image J software.

Western blot (WB) analysis

On the day of lysis (for time points see Immunocytochemistry (ICC)), cells were washed with ice-cold DPBS and then lysed with 1 mL lysis buffer (150 mM NaCl, 50 mM TRIS, 1% Triton X-100, protease inhibitor cocktail (Complete Mini®, Roche, Mannheim, Germany), phosphatase inhibitor (HALT, Pierce Biotechnology, Rockford, IL, USA) using a cell scratcher. Lysates were passed through a hypodermic needle (0.4 × 20 mm; Henry Schein Inc., Melville, NY; USA), vortexed, and kept on ice for 20 min, thereafter centrifuged for 10 min at 10,000 *g* and 4 °C, frozen in liquid nitrogen and stored at −80 °C until further analysis. Cell lysate total protein concentrations were determined using the RC DC™ Protein Assay Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). 2× Laemmli buffer (4% sodium dodecyl sulphate, 10% β-mercaptoethanol, 20% glycerol, 0,004% bromphenol blue, 0,125 M Tris HCl) was added to the samples, incubated for 5 min at 95 °C and briefly centrifuged. Samples were loaded in a Criterion™ XT 4–12% Bis-Tris gel in MOPS buffer (both Bio-Rad Laboratories, Inc., Hercules, CA, USA) and gel electrophoresis was run for 75 min. Afterwards, proteins were blotted on to a BioTrace polyvinylidene transfer membrane (Pall Corporation, Port Washington, NY, USA) for 30–40 min, applying 1 A and 25 V using the TransBlot Turbo (Bio-Rad Laboratories, Inc., Hercules, CA, USA). To avoid unspecific binding of antibodies, membranes were blocked using 5% BSA or 5% dry-milk in TBST, before incubation with the primary antibody (see Immunocytochemistry (ICC) and tubulin (1:2000; GeneTex, Irvine, CA, USA) or actin (1:4000; Millipore, Billerica, MA, USA) diluted in TBST at 4 °C overnight. The next day, membranes were thoroughly washed and incubated with the respective HRP-conjugated secondary antibody (1:2000 in TBST; goat-anti-mouse or goat-anti-rabbit; Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at room temperature. Signals were detected using enhanced chemo-luminescence assay (Amersham Life Science, Buckinghamshire, UK) exposed on Kodak X-OMAT X-ray films (Eastman Kodak Co., Rochester, NY, USA). Bands were analysed using the ImageJ software and normalised to tubulin or actin bands.

Bromodeoxyyuridine(BrdU)-assay

Proliferation was additionally analysed using the colorimetric immunoassay for the quantification of cell proliferation (BrdU-based; Roche Diagnostics, Mannheim, Germany). Procedures were conducted according to the manufacturer's instructions. Briefly, 1000 cells per well were seeded in a 96-well plate. After 2 days in PM, cells were treated according to the experimental groups described above for 2 h. BrdU labelling time was 6 h.

Statistics

Data are presented as mean ± standard error of the mean. The data was analysed for the normality of distribution using the Shapiro–Wilk or Kolmogorov–Smirnov-test and where appropriate repeated measure analysis of variance was used to check for differences over the time course of the experiment. To test for differences between the lactate concentrations used at each time point one-way

analysis of variance was applied. If significant differences were found, a post-hoc Bonferroni test was carried out. In case of not normally distributed data, the Mann–Whitney-*U*-test was used. Statistical significance was set at $p < 0.05$. Statistical analysis was carried out using IBM SPSS Statistics Version 19 for Windows (IBM SPSS Corporation, Chicago, IL, USA).

Results

Proliferation & apoptotic behaviour

Ki67 is expressed by cells which are in the G1/S/G2/mitotic phase of the cell cycle, but not in resting (G0) cells (Scholzen and Gerdes, 2000). Hence it is a reliable marker of proliferating cells. Fig. 1A shows the ratio of Ki67 positive cells to all cells analysed. After the first day of La treatment, proliferation ratios in the La treated samples decreased independently of the dose about 10-fold (10 mM La: 4.5 ± 1.7%; 20 mM La: 3 ± 1.5%) compared to control samples (0 mM La: 48 ± 5.7%; both $p < 0.001$). The following days, proliferation went down to approximately 5% in all conditions and remained around that level (data not shown).

Procaspase-3 is cleaved to the executioner act. Casp-3 (reviewed in Kiechle and Zhang, 2002). Therefore it allows identifying cells which are undergoing an apoptotic process (programmed cell death) and can thus be considered a marker of cellular stress. Densitometric analysis of cells stained for act. Casp-3 (Fig. 1C) showed that after the first La treatment bout, cells treated with 20 mM La DM showed a significant increase in act. Casp-3 compared to control and 10 mM DM (control vs. 20 mM La: $p = 0.004$; 10 mM vs. 20 mM La: $p < 0.001$). On day 2, overall activation went down in all conditions. Still, apoptotic induction was significantly higher in both La conditions compared to control DM treated cells (control vs. 10 mM La: $p = 0.007$; control vs. 20 mM La: $p < 0.001$). On day 3, apoptotic induction significantly decreased further in the control and 10 mM La cells compared with 20 mM La DM treated cells (control vs. 20 mM La: $p = 0.003$; 10 mM vs. 20 mM La: $p = 0.011$). Before the start of the treatment and after D3 no differences between the groups could be observed and apoptotic induction remained stable (data not shown).

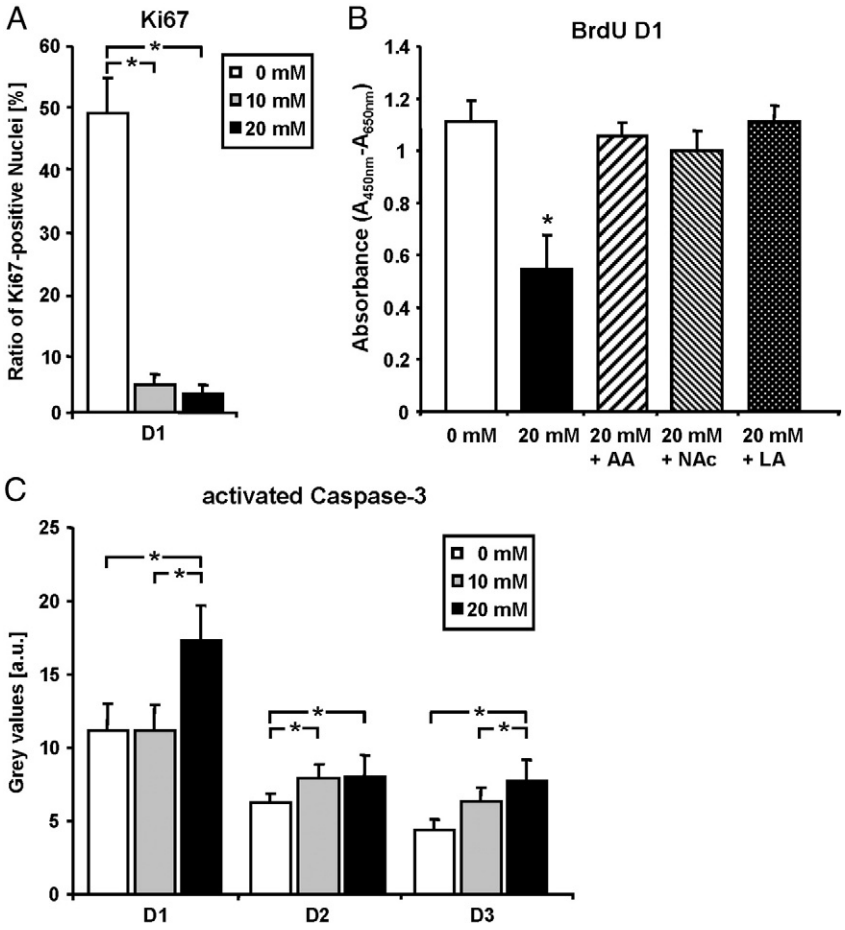

Figure 1 A. Incubation with La DM suppresses proliferation of C2C12 cells using Ki67 as a protein marker for cell proliferation. On day 1 the ratio of Ki67-positive cells is significantly lower in cells treated with 10 mM or 20 mM La DM compared to control DM. B. The proliferation suppression by La is also shown in the BrdU-assay and reversed by the addition of ascorbic acid (AA), N-Acetyl-L-cysteine (NAc), or linolenic acid (LA) C. Lactate induces apoptosis in C2C12 cells. Activated Caspase-3 was used as a protein marker for the initiation of apoptosis, i.e. cellular stress. (*) indicates statistical significance, i.e. $p < 0.05$.

Laconcentration-dependent differentiation behaviour

C2C12 cells were intermittently incubated with La DM with different physiologically relevant concentrations over a time period of 5 days simulating a microcycle of high intensity endurance training. La levels are associated with changes in pH. In our experiments we did not observe any significant pH changes between groups (data not shown). Therefore we consider the effects to be independent of pH. A screen of samples using WB analysis implied a partly dose-dependent La effect on several differentiation markers (Fig. 2). Pax7 content decreased in La compared to control samples on D1, with no differences on D3. On D5 the opposite was observed. Pax7 content was higher the higher the La concentration in the DM. The same observation on D1 was made for Myf5 (Fig. 2C). After that however and for all days for MyoD (Fig. 2D) no differences were observed. In contrast, with increasing La concentrations markers for late differentiation myogenin and MHC were less present in treated samples (Fig. 2E+F).

Myogenin is a marker for the onset of the end-terminal differentiation of myoblasts. Within a population of differentiating cells, its content will increase with time and start to disappear again when aggregation and fusion of myoblasts begin. In this study, the ratio of myogenin-positive nuclei in differentiating C2C12 cells was determined using ICC and results are shown in Fig. 3A+B and Supplemental Table 1. Number of myogenin-positive nuclei rose quickest in the control DM cells, peaking on day 3 of the experiment. The same level was reached when cells were treated with 10 mM La DM, but only on day 5. Myoblasts treated with 20 mM La did not reach that level at all during the 5 days of the experiment. WB results shown in Fig. 3C support the findings from the ICC.

MHC is part of the sarcomeric structure of skeletal muscle and occurs with the end-terminal differentiation of myoblasts into functional myotubes. Here, a pronounced concentration-dependent effect was observed (Fig. 4A+B; Supplemental Table 2). MHC was detected on D1 in the control and 10 mM La DM incubated cells, but not in the 20 mM La DM treated samples. Furthermore, 10 mM La DM resulted in significantly less nuclei lying within MHC-positive myotubes compared to

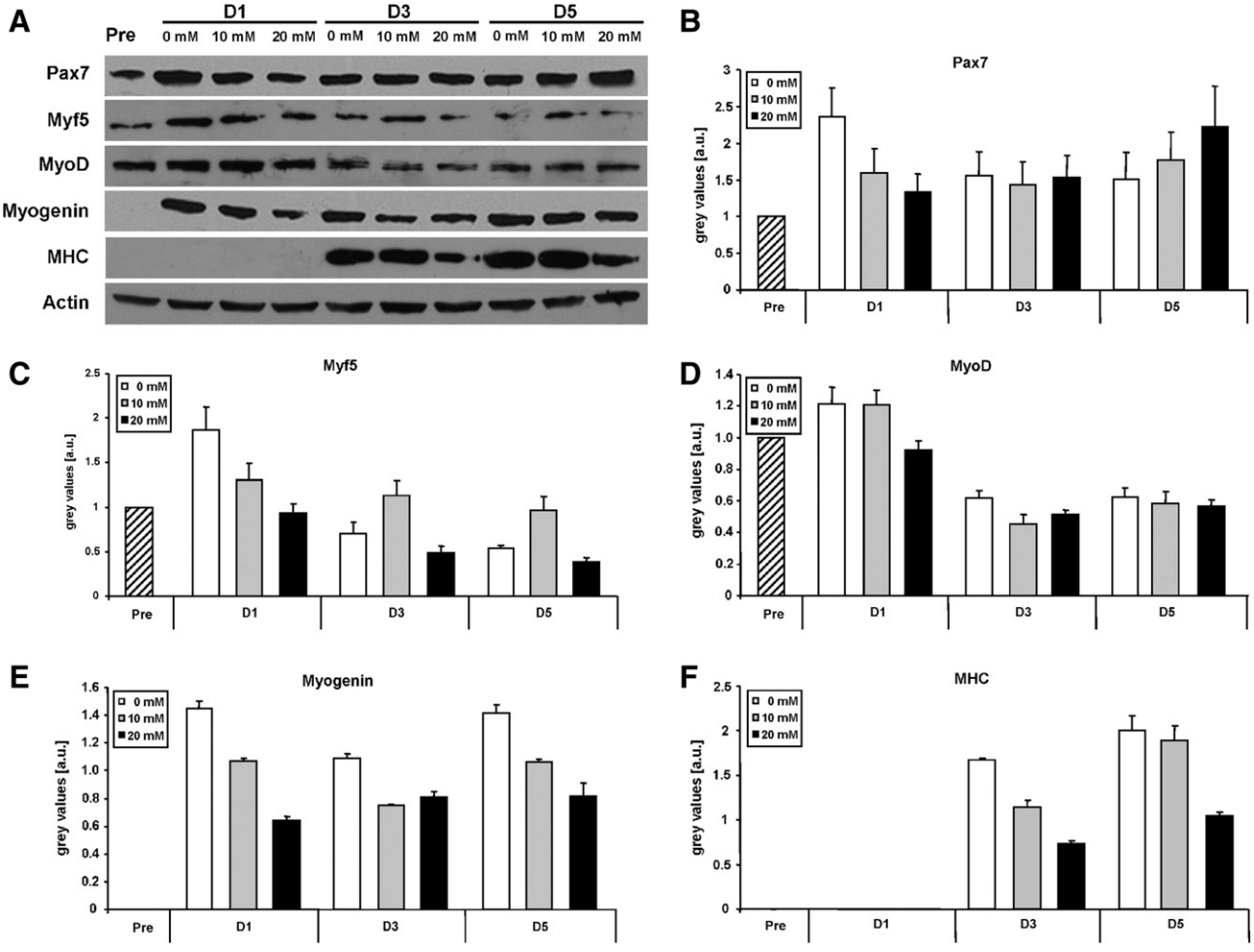

Figure 2 Incubation with La delays the differentiation process in a dose-dependent manner. Markers of early activation of SCs (Pax7, A) are less abundant in La-treated samples after 1 day, but more abundant after 5 days. In contrast, late differentiation markers (myogenin, MHC, E+F) are found to be less abundant with La treatment. A: Representative blots for Pax7, Myf5, MyoD, myogenin, and MHC. Actin was used as a reference. B-F: Graphical overview of the densitometrical analysis of Western blots.

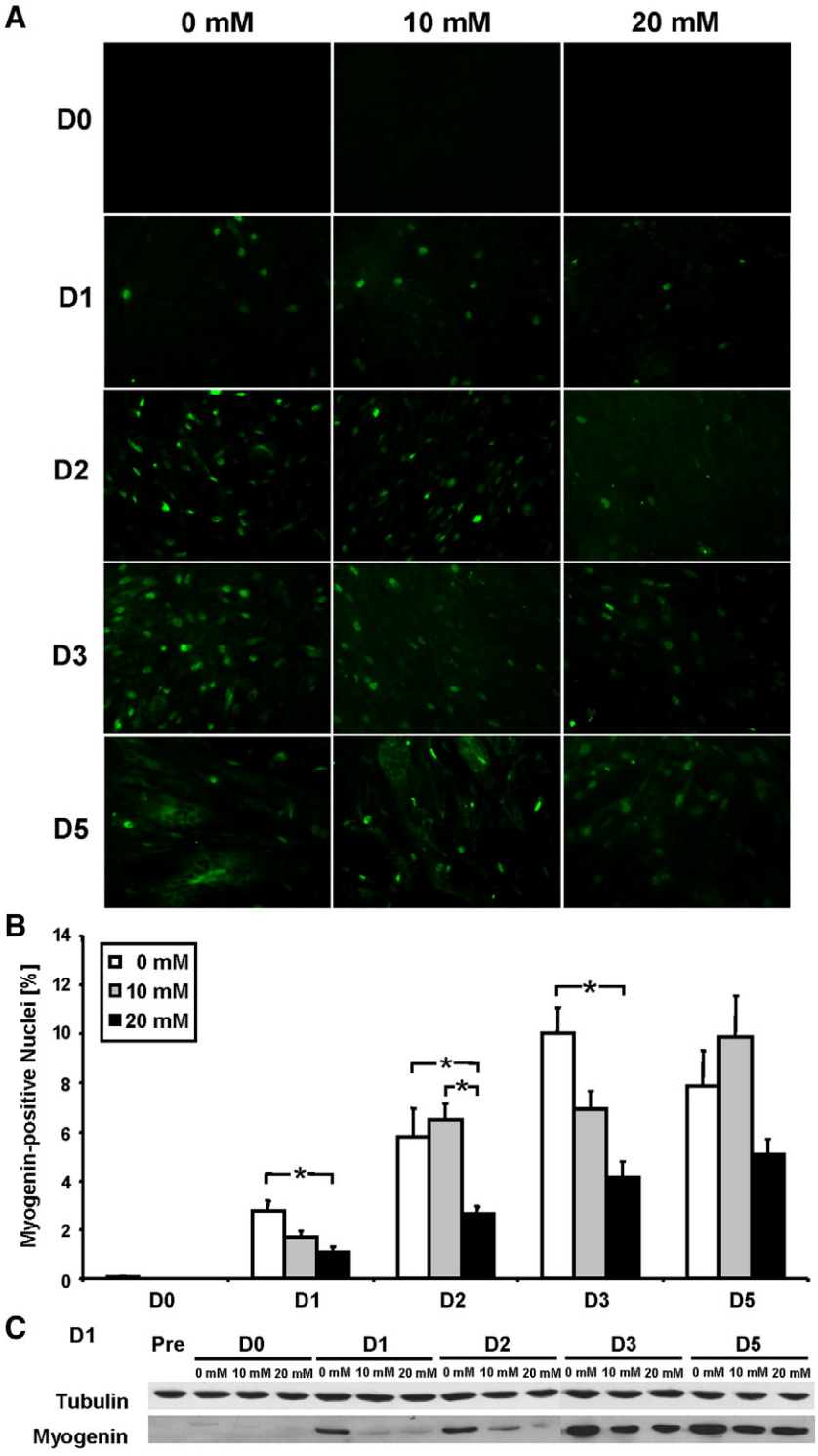

Figure 3 Immunocytochemical staining and Western blot analysis for myogenin of C2C12 cells over the time course of a 5 day differentiation experiment showed that myogenin occurs earlier in control than in La-treated samples. Additionally, 10 mM La DM samples show earlier myogenin occurrence than 20 mM La DM treated cells. A. Representative photographs of myogenin (green) staining on the respective days. B. Ratio of myogenin-positive cells on the respective days. C. Representative Western blots for myogenin and tubulin. (*) indicates statistical significance, i.e. $p < 0.05$.

control DM on day 1. The same pattern was found on day 2. As from day 3 on, no differences between the control and 10 mM La DM treated samples could be observed. 20 mM La samples showed significantly less nuclei within myotubes compared to 10 mM La and control DM on all days of the experiment. Again, results from the protein analysis via WB confirm these results (Fig. 4C). After 10 days of incubation (data not shown as control and 10 mM La DM treated cells started

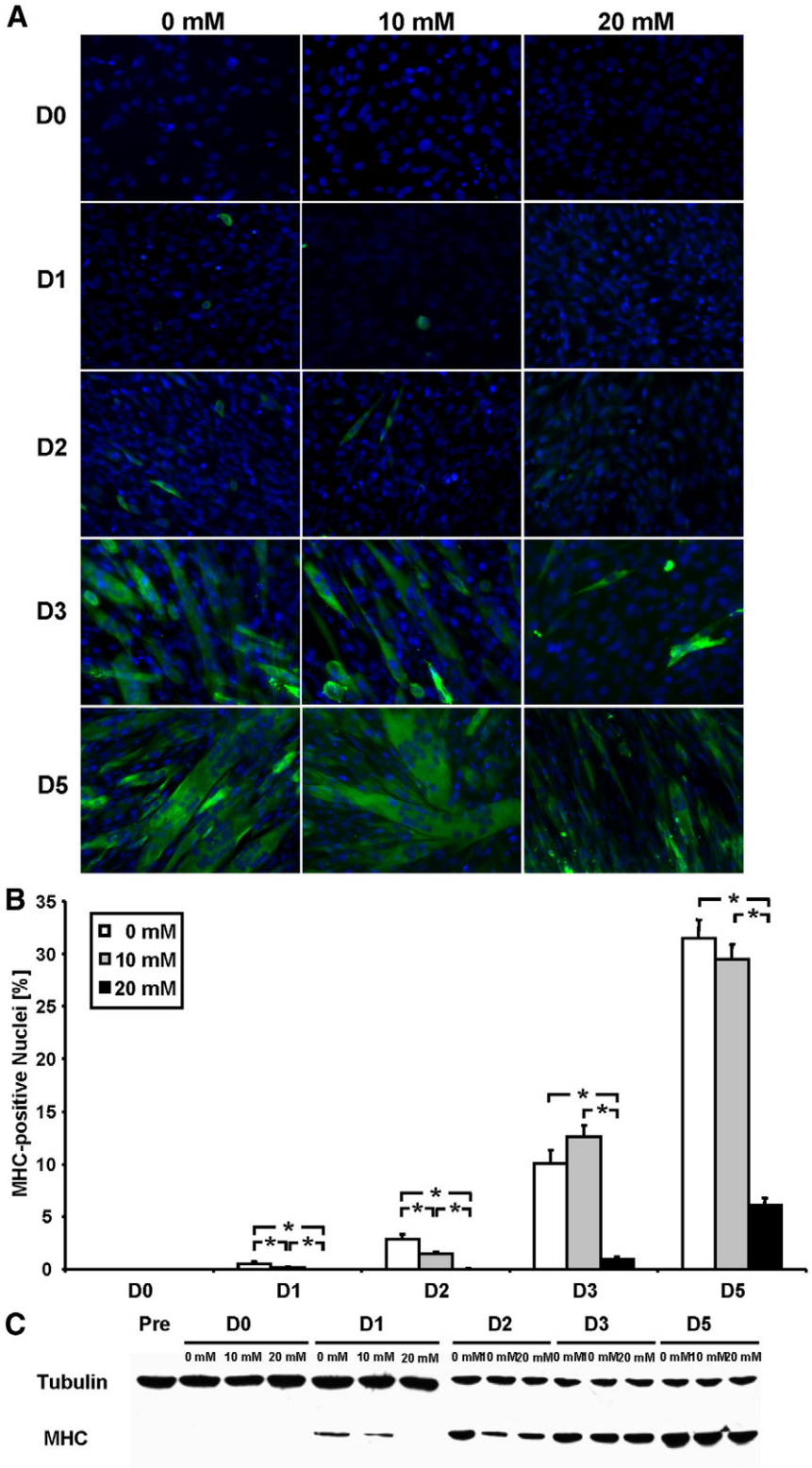

Figure 4 Immunocytochemical staining and Western blot analysis for MHC of C2C12 cells over the time course of a 5 day differentiation experiment showed that MHC occurs earlier in control than in the La-treated samples. A dose-dependent effect can also be observed. A. Representative photographs of MHC (green) and DAPI (blue) staining on the respective days. B. Ratio of MHC-positive myotubes on the respective days. C. Representative Western blots for MHC and tubulin. (*) indicates statistical significance, i.e. $p < 0.05$.

dedifferentiating and detaching), 20 mM were differentiated to the same extent as control and 10 mM La DM cells on day 5.

Summarising these data, cells treated with La differentiate to the same extent as control cells but at a later time point. Therefore we conclude that La temporarily delays the

differentiation process of C2C12 cells in a dose-dependent manner.

The role of ROS in the mediation of the La effect

A previous study suggested that the La effects are at least partly transduced within the cell by the shift of the redox potential, increased ROS production, and subsequent activation of MAPKs (Hashimoto et al., 2007). To further elucidate this assumption cells were treated with control, 20 mM La DM, and 20 mM La DM additionally containing different antioxidants and stained for 8-epi-PGF2α. This molecule is formed by a free radical-mediated, non-enzymatic peroxidation of arachidonic acid in membrane phospholipids and therefore a reliable marker to assess oxidative stress (Morrow et al., 1990). The results from this experiment are shown in Fig. 5. 8-epi-PGF2α was formed under all experimental conditions. However, 20 mM La DM showed by far the highest grey values whereas the addition of antioxidant agents led to control (LA) or lower (AA, NAc) levels of 8-epi-PGF2α.

So if the La effects are induced by the generation of ROS, the decrease in proliferation and the delaying effect on the differentiation of myoblasts should be at least reduced if not annihilated by the addition of antioxidants to the La DM. Conclusive data from these experiments are shown in Fig. 1B and Fig. 6 and Supplemental Table 3. A BrdU assay was performed to investigate the reversibility of the La-induced decrease in proliferation by different antioxidants. Results clearly show that 20 mM La DM administration significantly reduces proliferation rate of myoblasts (Fig. 1B) compared to all other conditions (20 mM La DM vs. control: $p = 0.02$; 20 mM La DM vs. 20 mM La + AA: $p = 0.018$; 20 mM La DM vs. 20 mM La + NAc: $p = 0.031$; 20 mM La DM vs. 20 mM + LA: $p = 0.015$) demonstrating that AA, NAc, and LA were indeed able to reverse the La effect on the proliferation.

Furthermore, results from the experiments investigating the differentiation effects show that C2C12 cells treated with 20 mM La DM elicited a significantly higher ratio of Pax7-positive nuclei than control treatment (Fig. 6C). This effect was annihilated by the addition of AA to the 20 mM La DM. However, the lower ratio of Pax7-positive cells was not significant for 20 mM La DM + NAc and 20 mM La DM + LA. Overall, these findings were confirmed using WB analysis (Fig. 6D). Less clear are the results from the Myf5 analysis. Overall, a tendency for a La effect can be seen in ICC and WB (Fig. 6E+F), but the differences were significant only between 20 mM La DM and 20 mM La DM + AA treatment in the ICC. The most distinct results were attained from the myogenin and MHC staining. Here, the La effect was

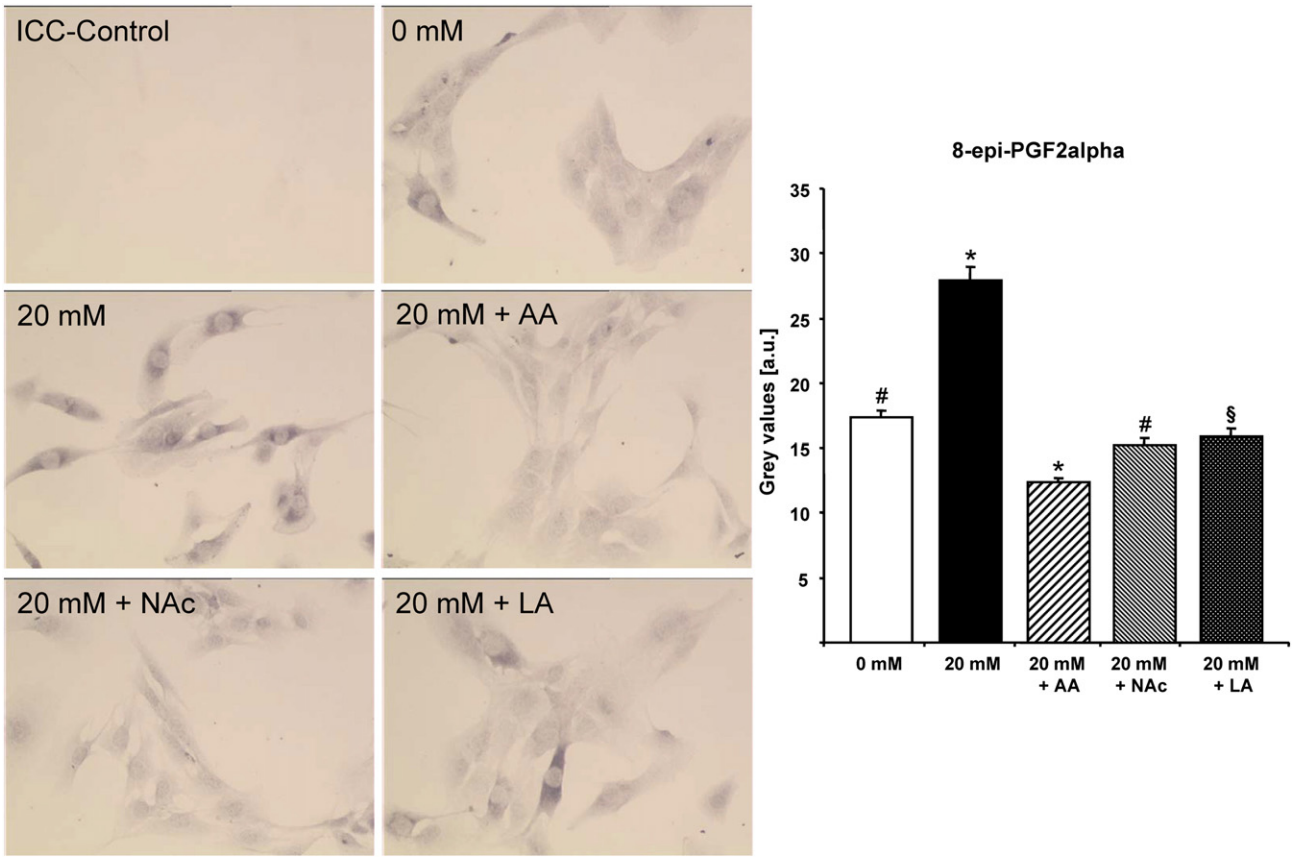

Figure 5 Immunocytochemical staining of treated cells for 8-epi-PGF2α as a marker of oxidative stress. Cells treated with 20 mM La DM were exposed to high levels of oxidative stress. This effect was negated when antioxidants (ascorbic acid AA, N-Acetyl-L-cysteine NAc, linolenic acid LA) were added to the 20 mM La DM. (*) indicates statistical significance, i.e. $p < 0.05$, compared to *all other conditions*. (#) indicates statistical significance, i.e. $p < 0.05$, compared to *all other conditions except LA*. (§) indicates statistical significance, i.e. $p < 0.05$, compared to *20 mM La DM and 20 mM La DM + AA*.

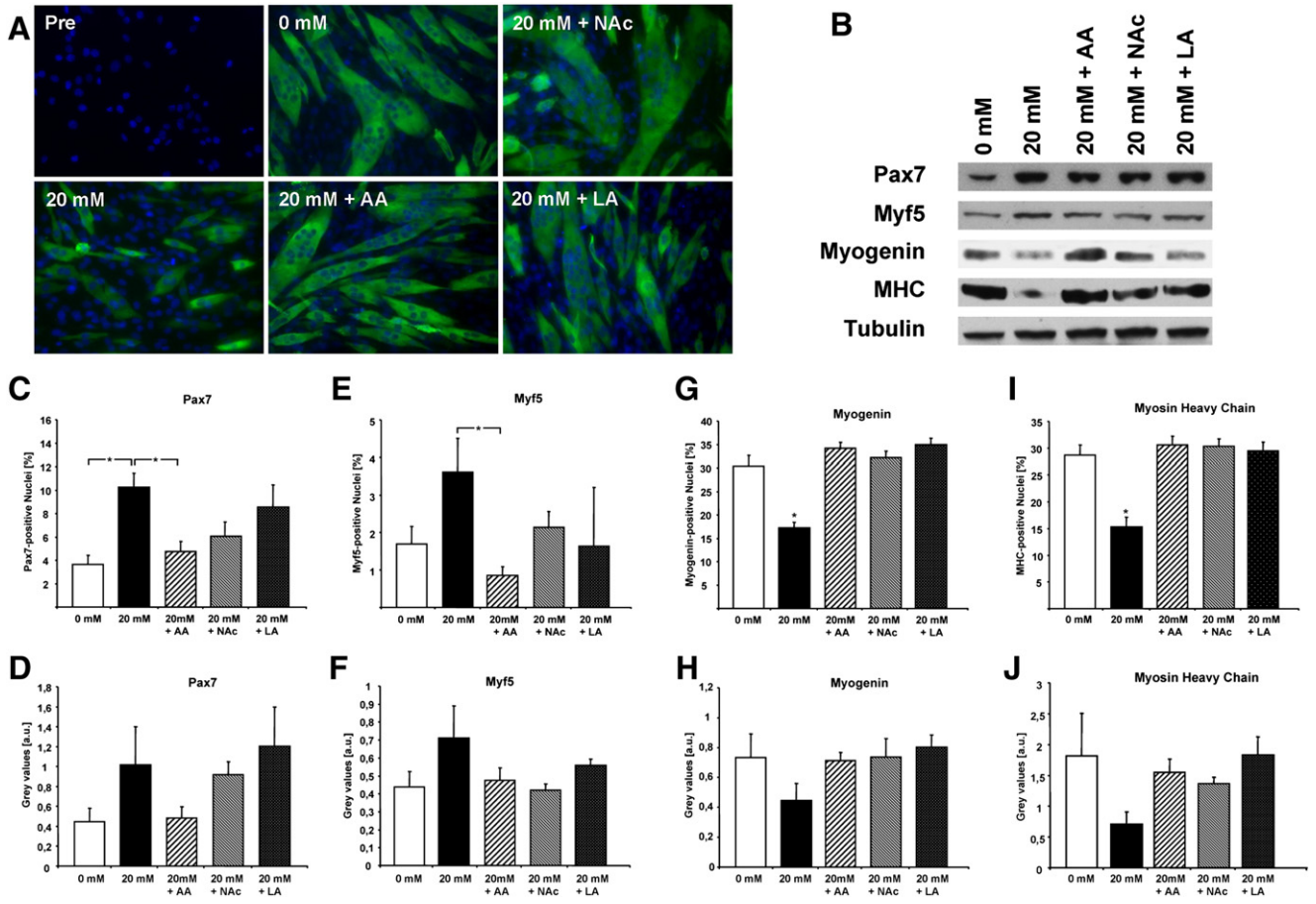

Figure 6 Immunocytochemical staining and Western blot analysis for Pax7, Myf5, myogenin, and MHC of differentiating C2C12 myoblasts treated intermittently with control DM, 20 mM La DM or with 20 mM La DM containing the different antioxidants ascorbic acid (AA), N-Acetyl-L-cysteine NAc, or linolenic acid (LA) for 5 days. A. Images of MHC-staining in myoblasts before (Pre) and after 5 days of incubation with the respective DM. B. Representative Western blots for Pax7, Myf5, myogenin, and MHC. Tubulin was used as a loading control. C-J: Graphs representing the analysis of the ICC (C, E, G, I) and Western blots (D, F, H, J) for the analysed proteins Pax7 (C, D), Myf5 (E, F), myogenin (G, H), and MHC (I, J). (*) indicates statistical significance, i.e. $p < 0.05$, compared to the indicated experimental group (bars). If no bars are shown then significance is given to all other experimental groups.

clearly observed in the 20 mM La DM incubated cells where compared to control conditions the number of myogenin- and MHC-positive cells was significantly reduced. This effect was completely reversed by all of the applied antioxidants in both the ICC (Fig. 6G+I) and the WB (Fig. 6H+J).

Discussion

The C2C12 cell line is a subclone from a myoblast line obtained from the thigh muscle of adult C3H mice muscle following crush injury. These cells are capable of rapid differentiation upon serum withdrawal and give rise to contracting myotubes expressing muscle-specific proteins. Hence, these cells are a strong model for studying the proliferation and differentiation of activated SCs which are the skeletal muscle tissue-specific stem cells.

The primary novel and most important finding of this study is that La induces withdrawal from the cell cycle and early differentiation of C2C12 cells, but delays late differentiation in a timely and dose-dependent manner. In cell culture experiments permanent withdrawal from the cell cycle (as necessary for early differentiation) is due to serum deprivation (Yaffe and Saxel, 1977b; Kitzmann et al., 1998) leading to changes in cell cycle regulators (Kitzmann and Fernandez, 2001). Hence, one would expect a reduction in proliferating cells after the medium switch. However, data from both the Ki67-analysis as well as the BrdU assay show that proliferation rates in control cells are much higher. Therefore we conclude that La enhances the changes in cell cycle regulation induced by serum withdrawal. Furthermore, a delay of the appearance of differentiation markers is reported here. Pax7 and Myf5 are increased with La treatment, whereas markers for the late differentiation phase (myogenin and MHC) are decreased indicating persistence of early differentiation phase of C2C12 cells after 5 days of La treatment. Hence, our data suggests that La is additionally a potent regulator of gene expression during C2C12 myogenesis.

The second novel finding is that La induces oxidative stress within C2C12 myoblasts. Our observations clearly demonstrate that 2 h incubation with 20 mM La DM induces 8-epi-PGF2α levels to rise. Adding AA, NAc, or LA to the 20 mM La DM leads to a reduction of 8-epi-PGF2α levels, additionally establishing that ROS formation by La can be

reversed by the use of antioxidants. Interestingly, the areas around the nuclei were mostly affected by oxidative stress where mitochondrial density is highest within the cell. We therefore additionally argue that La leads to increased ROS formation mostly within the mitochondrial membranes. This notion is supported by our finding that AA and NAc showed the highest efficacy of ROS scavenging. AA and NAc, in contrast to LA, unfold their direct antioxidative capacity mainly in the mitochondrion (Banaclocha, 2001; Nordberg and Arnér, 2001; Moreira et al., 2007; Mandl et al., 2009; Gillissen, 2011). Both substances were able to reduce the oxidative stress even below control levels. The mechanism by which LA deploys its antioxidative capacity remains elusive, but it has been described to act by the upregulation of the antioxidative enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (Cat) (Yu et al., 2013) which suggests a time-delayed, and hence a weakened effect for the time period observed. Nonetheless, further experiments are necessary to provide clear evidence of the idea that mitochondrial ROS generation is essentially elevated by La. The La-induced formation of ROS is controversially discussed in the literature. On the one hand, La was shown to be a capable scavenger of ROS in the absence of cells (Anbar and Neta, 1967; Groussard et al., 2000; Lampe et al., 2009). Whereas this finding was shown to be also present in cultured hepatocytes (Groussard et al., 2000), a protective effect was not established in neuronal precursor cells (Lampe et al., 2009). In contrast, other studies demonstrated that La increases ROS formation. One report describes the La-dependent enhancement of hydroxyl radical generation by the Fenton-reaction in a cell-free model (Ali et al., 2000). Hashimoto et al. (2007) showed an increased production of H_2O_2 in L6 myoblasts by 20 mM La incubation indicating increased oxidative stress which is in agreement with our findings. Furthermore, the results for act. Casp-3 imply that La is able to induce apoptotic events, i.e. cellular stress. Whereas no data on the ability of La to induce the apoptotic pathway in C2C12 exists, ROS have been described to be able to initiate programmed cell death in this type of cells (Nishida et al., 2007; Gilliam et al., 2012; Lee et al., 2013). Our results unambiguously show that with La treatment, the oxidative stress increases within the cells. Therefore the rise in cleaved act. Casp-3 within cells is not surprising and provides further evidence for the conclusion that La induces cellular stress, marked by the increased generation of ROS.

Thirdly we can report that La-induced effects are ROS-dependent and can therefore be reversed by the addition of antioxidants such as AA, NAc, and LA. Although no data is available on the effects of La on C2C12 differentiation, numerous studies exist elucidating the question how ROS influence this process. One report shows that the ROS-induced activation of NF-κB/iNOS pathway is necessary for differentiation (Piao et al., 2005). Another report from the same group implies that certain endogenous ROS concentrations are essential in differentiating myoblasts and that the addition of antioxidants to the cells resulted in differentiation inhibition. In contrast, Cyclosporin A-induced ROS generation has been shown to block myoblast differentiation in early myoblasts. The conclusion was that the addition of Cyclosporin A to differentiating cells led to toxic levels of ROS, blocking muscle differentiation even when antioxidants were added (Hong et al., 2002). Other studies provide evidence for a negative effect of ROS on myoblast differentiation. Langen et al. described that oxidative stress per se is sufficient to block myogenic late differentiation (Langen et al., 2002). H_2O_2 administered in different concentration (20–200 μM) reduced myogenin and MHC protein content, creatine kinase activity as well as troponin I gene transcription all in a dose-dependent manner and that the inhibition of myotube formation was reversible when NAc was added to the culture medium (Langen et al., 2002). Furthermore it was demonstrated that 25 μM H_2O_2 markedly reduced Myf5 and muscle regulatory factor 4 gene expression 2–3-fold, whereas myogenin was even 60-fold down-regulated (Hansen et al., 2007). This clearly supports the idea that myogenin expression, and therefore late myogenesis is very susceptible to oxidative stress, and thus La-sensitive. Applying 1 mM H_2O_2 to C2C12 cells decreases Myf5 and MHC gene expression (Furutani et al., 2009). Taken together, the literature supports the notion that H_2O_2 can have different outcomes depending on the dose (Powers et al., 2010). However, regarding the data available for late differentiation markers as well as the findings on the reversibility of the La-effect by the addition of anti-oxidants, we conclude that the La-induced timely delay of late differentiation is mediated via ROS.

Applying these key findings to an in vivo situation, the data imply that common views on training design and periodisation (as well as the prescription of antioxidant nutritional supplements) should be reassessed in order to accelerate muscle adaptation.

We suggest that one should start with a type of training to increase the SC pool most efficiently. Several human studies reported an increase in SC number of over 80% 5–8 days after a single bout of maximal eccentric contraction (Crameri et al., 2004, 2007; O'Reilly et al., 2008). In contrast, other studies applying an endurance type training (Charifi et al., 2003; Verney et al., 2008) or lower intensity resistance training (Verney et al., 2008; Kadi et al., 2004; Mackey et al., 2007, 2011) in humans could only demonstrate markedly smaller changes in SC pool expansion of 30–62.5%. The next cycle within the training regimen should lead to the withdrawal from the cell cycle and promotion of early differentiation. Considering the data reported here, a high intensity endurance training cycle accompanied by high La levels should be an efficient way to drive proliferating SCs to withdraw from the cell cycle and into the early differentiation phase. Although several molecular promoters (Millay et al., 2013) and involved signalling pathways (reviewed in Hindi et al., 2013) of myoblast fusion were identified, no data is available on how different types of exercise modify this process. However, it should not be accompanied by high La levels as our data implies that these prevent further differentiation and fusion of myoblasts with already existing myofibres, eventually suppressing hypertrophy.

Our findings furthermore suggest that the use of antioxidants as nutritional supplements could influence the process of skeletal muscle adaptation, possibly even in a negative way. However, advising on nutritional supplements needs precaution due to its complicated nature and is not further discussed here.

Conclusion

In summary, La plays a regulatory role in the myogenesis of C2C12 myoblasts in vitro. It leads to cell cycle withdrawal

and promotes early differentiation, but is not able to facilitate late differentiation. From our data we can furthermore conclude that La uses a ROS-sensitive signalling network to mediate its effects as the addition of antioxidants reverses these. Development of exercise regimens should take these observations into account. Nevertheless, further research is necessary to elucidate which sequential pattern and timing of training stimuli generates the best possible response, i.e. yields the most effective training programme.

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